REMARKS

Claims 74-92 are pending and under examination. Applicants have hereinabove amended claim 74. Support for the amendments to claim 74 can be found in Figure 12, and in the specification as originally filed at, *inter alia*, page 32, lines 7 to 23; and at page 48 lines 26-29. In addition, applicants have amended the first paragraph of the specification to delete the reference to incorporation by reference. Accordingly, applicants maintain that this Amendment raises no issue of new matter, and respectfully request entry of this Amendment. After entry of this Amendment, claims 74-92 will still be pending. In view of the amendments made herein and the remarks below, applicants respectfully request that the Examiner's objections and rejections be withdrawn.

Objection to the Specification

The Examiner stated that the specification is objected to as documents have been improperly incorporated by reference.

In response, applicants respectfully traverse the Examiner's rejection. However, in order to expedite prosecution, but without conceding the correctness of the Examiner's position, applicants have hereinabove amended the specification to delete reference to incorporation of these cited references. Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this objection.

Rejection of Claims Under 35 U.S.C. §112 (Written Description)

The Examiner stated that claims 74-92 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The Examiner also stated that the specification, however, cautions artisans "in order to obtain accurate measure of the mass of the sequencing DNA fragments, the samples must be free from alkaline and alkaline-earth salts. Samples must be desalted and free from contaminants before the MS analysis." The Examiner further stated that a review of the specification, including passages cited by applicant, fails to find an adequate written description of where mass spectrometry is performed on the DNA sequencing fragments where the sample contains any of the above-noted contaminants.

In response, applicants respectfully traverse the Examiner's rejection. Initially, applicants note that to fulfill the written description requirement, the specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention (MPEP §2163(I)). Applicants note that the claimed method is for sequencing DNA, that the essential steps required to do so are described and set forth in the claims and, moreover, the method works as proven by the working examples. The Examiner's objection, however, is that the claimed method encompasses sequencing samples not free of alkaline salts, which (as applicants' specification states) can be problematic for obtaining sequence data. However, applicants' specification also specifically states that the claimed method, because of a purification step where all other components besides DNA sequencing fragments terminated with a biotinylated

dideoxynucleotide are washed away, i.e. alkaline salts are washed away (see page 37 and page 41, lines 9-11), eliminates this problem. Accordingly, the specification itself specifically raises the alkaline salt issue, and, moreover, describes how the alkaline salt problem is eliminated. Applicants direct the Examiner's attention to steps c) and d) of claim 74 which provide an affinity step, and a washing step, respectively. Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this objection.

Additionally, applicants understand the Examiner's position to be that the problem of alkaline earth salts is not overcome because the working examples do not specifically state the samples contain alkaline salts. However, the Examiner has offered no reasoning or art to suggest how the claimed method, which the specification states is specifically designed to overcome this problem (amongst others), would somehow not eliminate the salts.

The Examiner further stated that the claimed method has been interpreted as comprising a plurality of wells connected via a channel, where the channel and wells are within a chip. The Examiner further stated that a review of the disclosure, however, fails to find an adequate written description of such a device, rather, the specification has been found to provide a description via Fig. 12, of two 96-well plates that are connected via glass capillary tubes to corresponding single channels in a chip.

In response, applicants respectfully traverse the Examiner's position. Applicants note that the Examiner has suggested an embodiment not explicitly described in the specification wherein the channel and wells "are within a chip". Applicant notes that

the claimed method, however, provides "a system comprising (i) a channel [], wherein the channel comprises two ends, (ii) a plurality of wells [], (iii) a connection between one end of the channel and a first well, and a connection between the other end of the channel and a second well." Such a working system is described in the specification at page 48, lines 15 to 33, and in Figure 12. This description satisfies the written description requirement. Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Applicants further note that the Examiner's rejection on this point actually appears to be an enablement rejection, but further note that it is not necessary to describe every imagined embodiment (see MPEP 2164.02, last paragraph). For example, one imagined embodiment of the claimed method, analogous to the Examiner's, may be wherein the described system is contained "within a glass beaker". However, a glass beaker need not be described in the specification in order that the written description or enablement requirement be satisfied.

The Examiner further stated that the device described lacks any means for applying pressure such that any one, much less 96 different samples could be passed through the coated channels in one direction, much less back-and-forth, thereby permitting/enabling the binding of the DNA sequencing fragments.

In response, applicants respectfully traverse the Examiner's position. More particularly, as previously observed, applicants note that moving a liquid through a channel is a fundamental technique which applicants maintain would be well known to those of ordinary skill in the art. For example, see use of a syringe

pump to move fluids through a channel for DNA sequencing on page (iii) of Pang and Yeung, **Exhibit A**. Applicants further note that the level of ordinary skill in the art (of DNA sequencing) is high, and that "information which is well known in the art need not be described in detail in the specification", MPEP §2163 (II)(A)(2). Accordingly, applicants maintain that the claimed invention is clearly described in the specification, and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

The Examiner further stated that the claimed method has been interpreted as requiring but a single pass of the sample through the channels. However, page 48, lines 26-29, describes a method requiring pressure to be applied in reverse in order to drive "the sample through the channel multiple times," thereby ensuring a high efficiency solid phase capture.

In response, applicants respectfully traverse the Examiner's position. More particularly, applicants note that the specification support cited by the Examiner regarding multiple passing of the sample through the channel merely shows how the efficiency of the system can be increased, but that it is not a prerequisite for the system to function. However, in order to expedite prosecution, but without conceding the correctness of the Examiner's argument, applicants have hereinabove amended claim 74 to recite a means for moving the sample by pressure multiple times through the channel between wells. Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

The Examiner stated that the claimed method has also been

interpreted as encompassing the simultaneous sequencing of multiple DNA sequencing fragments in a common channel. The Examiner stated that to perform such a maneuver would present situations where multiple signals would be generated at the same time, yet would correspond to the different templates, and that the use of different DNA fragments will cause situations where the nucleotide sequence is anything but clearly resolvable.

The Examiner also stated that the claimed method fairly encompasses the use of mass spectrometry in the analysis of the DNA fragments, but that the use of lasers in performing mass spectrometry is recognized in the art as causing significant problems in sequencing. The Examiner stated that in support of this position attention is directed to US Patent Application Publication 2002016842A1, paragraph 13.

In response, applicants respectfully traverse the Examiner's rejection. Initially, applicants note that the mass resolution of the claimed method is sufficient to distinguish the different fragments by mass spectrometry. In response to the Examiner's assertion that "attorney argument is not evidence" (which, as cited by the Examiner, refers to non-obviousness rebuttal argument's) applicants note that they are merely pointing out in other words what is already explicitly stated in the specification and shown in the working examples (see figure 2, and page 38, lines 27 to 31). The working examples distinguishing different fragments using MALDI-TOF as described in the specification are evidence that the claimed method is sufficient to distinguish the fragments. In addition, without conceding the correctness of the Examiner's position, but in order to expedite prosecution, applicants have hereinabove amended claim 74 to

state that detection is performed by MALDI-TOF mass spectrometry. Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

The Examiner further stated that the specification is essentially silent as to how one is to resolve any conflict that results when the signals are derived from a plurality of fragments when the fragments are of equal length yet are from different templates.

In response to this, applicants note that the claimed method is directed to sequencing a DNA and that the DNA (template) is contacted with the appropriate components for fragment generation.

Applicants therefore maintain that the claimed invention satisfies the written description requirement, and respectfully request that the Examiner reconsider and withdraw this rejection.

Rejection of Claims Under 35 U.S.C. §112 (Enablement)

The Examiner stated that claims 74-92 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. The Examiner stated that, as presented above, the specification has not been found to provide an adequate written description of the invention to where the specification does not reasonably suggest that applicant did not possess the entire invention at the time of filing, and that it is well settled that one cannot enable that which they do not yet possess. The Examiner also stated that the record shows that the claimed method fairly encompasses embodiments where art-recognized issues of enablement would be encountered, yet the

specification is effectively silent as to how they are to be overcome without the skilled artisan resorting to undue experimentation.

In response, applicants respectfully traverse the Examiner's rejection. Applicants first note that the standards for satisfying the written description and enablement requirements are distinct, and that the ability of one skilled in the art to practice the claimed invention without undue experimentation is the focus of the enablement requirement. Nevertheless, applicants have hereinabove pointed out how the claimed invention is fully described in the specification, and since the reasons supporting the enablement rejection are the same as those supporting the written description rejection, these arguments apply to the enablement rejection.

In response to the Examiner's statement regarding "art-recognized issues of enablement", applicants maintain that the claimed method presented here is demonstrated in the working examples provided, where different fragments are sequenced using MALDI-TOF mass spectrometry. Applicants note that the issues that arise in the <u>different</u> methods discussed in prior art cited by the Examiner as hypothetically applying to the claimed method are not borne out by the empirical results actually obtained using the claimed method and set forth in the specification (as described above).

Accordingly, applicants maintain that the claimed invention is enabled by the specification, and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Summary

Applicants maintain that the claims pending are in condition for allowance, and accordingly, allowance is respectfully requested.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorneys invite the Examiner to telephone them at the number provided below.

No fee is deemed necessary in connection with the filing of this Amendment. If any fee is required, however, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450

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Automated one-step DNA sequencing based on nanoliter reaction volumes and capillary electrophoresis

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ABSTRACT

An integrated system with a nano-reactor for cycle-sequencing reaction coupled to on-line purification and capillary gel electrophoresis has been demonstrated. Fifty nanoliters of reagent solution, which includes dye-labeled terminators, polymerase, BSA and template, was aspirated and mixed with the template inside the nano-reactor followed by cycle-sequencing reaction. The reaction products were then purified by a size-exclusion chromatographic column operated at 50°C followed by room temperature on-line injection of the DNA fragments into a capillary for gel electrophoresis. Over 450 bases of DNA can be separated and identified. As little as 25 nl reagent solution can be used for the cycle-sequencing reaction with a slightly shorter read length. Significant savings on reagent cost is achieved because the remaining stock solution can be reused without contamination. The steps of cycle sequencing, on-line purification, injection, DNA separation, capillary regeneration, gel-filling and fluidic manipulation were performed with complete automation. This system can be readily multiplexed for high-throughput DNA sequencing or PCR analysis directly from templates or even biological materials.

INTRODUCTION

For sequencing the human genome, capillary electrophoresis (CE) array systems (1–7) have minimized the bottleneck involved in separating the Sanger fragments. Commercially available CE array systems (8–10) have advanced the human genome project significantly. It has been estimated that the entire human genome could be sequenced by the end of 2000. DNA sequencing speed has improved tremendously. However, cost savings on the reagents for cycle-sequencing reaction has not been realized even though CE requires very small amounts of sample for separation and detection. Ultra small volume sample preparation is needed to reduce the usage of DNA templates and reagents, leading to a substantial reduction in the cost per base sequenced. The coupling of automated cycle-sequencing reaction with highly multiplexed capillary array electrophoresis has the potential to further reduce the cost in large-scale DNA sequencing projects.

Miniaturization of cycle sequencing in a glass capillary (11–14) has been demonstrated at the microliter level. Moving the cycle-sequencing reaction into a capillary has the additional advantage of increasing the reaction speed due to the small heat capacity of a capillary versus a heating block or a water bath. A capillary reactor is also compatible with highly multiplexed electrophoresis in a parallel capillary array. Several groups (15,16) have demonstrated on-line reaction in a capillary coupled with electrophoretic separation. Multiplexed systems have also been demonstrated (17–19). However, they did not exploit the small amount of DNA sample required for CE separation.

In standard reactions, a thermal block is used to control the temperature profile. However, because of the large mass, the transition time is slow. Numerous heating methods have been demonstrated to speed up the temperature change in the PCR or cycle-sequencing reaction. Hot air (20) was successfully coupled with capillaries to provide rapid temperature change. A resistive thin film (21,22) put on capillaries or microchips has also been demonstrated successfully. Infrared heating (23) has also been used for rapid PCR reaction.

Although robotics has provided advantages in repetitive operations with high precision for solution manipulation, the adaptation to highly multiplexed capillary array separation and detection suffers from many incompatibilities in terms of the total reaction volume, purification by centrifugation, lyophilization, and sample injection after reconstitution and denaturation. Standard technology calls for 10 µl sample volumes for cycle sequencing. State-of-the-art workstations allow the use of sample volumes of 1 µl (11–13). The cost related to reagents, dye labels, buffers, etc., could become negligible when <100 nl reaction volume is used. The real issue is whether one can prepare small volume solutions and deliver these to the reaction zone for the cycle-sequencing reaction, purify (if necessary) and then inject them for CE separation. If one needs to pre-mix the reagent with the template on a microliter scale before introducing into the reaction zone, large amounts (1 µl) of reagent would be wasted even when only a small volume (50 nl) is injected into the capillary for electrophoresis.

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A microchip providing integrated operation from Sanger reaction to sequencing separation is a promising approach (21,24,25). For a nanoliter scale reaction, Burns *et al.* (21) demonstrated that discrete 120 nl drops can be metered from each channel, mixed together and positioned in the reaction zone with the use of hydrophobic patches and an air-pressure source in a microchip. Only DNA amplification followed by gel electrophoresis but not the more demanding case of DNA sequencing was demonstrated. Soper *et al.* (14) showed that the DNA template could be bound to the capillary wall via biotin–streptavidin–biotin linkage while the reagent was pumped into the reaction zone through electroosmotic flow. Solid-phase PCR and cycle-sequencing reaction followed by CE were demonstrated. Since dye-labeled primer cycle-sequencing reaction was performed, no purification was required. As little as 62 nl reaction volume has been achieved although the actual amount of reagents used is close to 5 µl. Also, no complete DNA sequencing result was shown because only one primer-labeled cycle-sequencing reaction was performed. Evenson *et al.* (26) demonstrated that by using a piezo-ceramic actuator one can rapidly mix two 1 µl solutions in under 3 s inside a capillary. However, that system may pose a challenge for multiplexed operation.

The work presented herein describes an automated nano-reactor for cycle-sequencing reaction with on-line size exclusion chromatography (SEC) purification and capillary gel electrophoresis. As little as 25 nl reagent volume was required. A simple procedure allows the reagent solution to mix with the template solution inside the nano-reactor. By using this protocol, the bulk reagent solution can be re-used without contamination. This provides real cost savings based on the amount of reagents used. A simple and inexpensive flexible heater design for the nano-reactor allows for future scale up for capillary-array DNA sequencing.

MATERIALS AND METHODS

Reagents, buffers and separation matrix

TE buffer solution (1×) was purchased from Amresco (Solon, OH). Bovine serum albumin (BSA) (10×) (0.25 µg/µl) was obtained from Idaho Technology Inc. (Idaho Falls, ID). TBE with 7 M urea buffer (1×) was prepared by dissolving pre-mixed TBE buffer powder (Amresco, Solon, OH) and urea (ICN Biomedicals Inc., Aurora, OH) in deionized water. The capillary coating matrix was made by dissolving 2% (w/v) of 1 300 000 molecular weight poly(vinylpyrrolidone) (PVP) (Sigma, St Louis, MO) into deionized water. Tris, HCl, $MgCl_2$ and poly(ethylene oxide) (PEO) were from Aldrich Chemical (Milwaukee, WI). The sieving matrix was an entangled polymer solution made by dissolving 1.5% (w/v) of 8 000 000 molecular weight PEO and 1.4% (w/v) of 600 000 molecular weight PEO in 1× TBE buffer with 7 M urea. The solution was stirred vigorously overnight by a stirring bar until all the material was dissolved and no bubbles visible. Even smaller bubbles may remain but these pose no difficulties in capillary electrophoresis. The bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ) for DNA separation was 360 µm outer diameter (o.d.), 75 µm inner diameter (i.d.) and 59 cm long with 50 cm effective length. It was flushed with TBE buffer with urea followed by 2% PVP solution before introduction of the PEO sieving matrix.

Sequencing reaction protocol

Either ABI PRISM dye terminator cycle-sequencing ready reaction kit with AmpliTaq DNA polymerase, FS (ABI, Foster City, CA) or ThermoSequenase dye terminator cycle sequencing pre-mix kit (Amersham Life Science, Cleveland, OH) was used. M13mp18 (0.05 µg/µl) ssDNA (Amersham Life Science) in 50 mM Tris, 2.5 mM HCl, 2 mM $MgCl_2$ and 1× BSA as stock solution was used. The reaction mixture for AmpliTaq FS polymerase consisted of 8 µl of terminator ready reaction mix, 3.2 pmol universal –21 M13 primer and 2 µl 10× BSA. The reaction mixture for ThermoSequenase consisted of 8 µl of terminator ready reaction mix, 5 pmol universal –17 M13 primer and 2 µl 10× BSA. These were prepared in advance and a 0.5-µl aliquot was used for a series of reactions.

The temperature protocol for the AmpliTaq cycle-sequencing reaction was as follows: the reaction mixture was heated to 95°C and held for 2 min; 35 thermal cycles were performed with denaturation at 96°C for 10 s, annealing at 50°C for 15 s and extension at 60°C for 4 min. The sample was then ramped to 95°C and held for 2 min. When Thermo-Sequenase cycle-sequencing reaction was used, the annealing temperature was adjusted to 45°C.

Instrumentation

Figure 1 shows the schematic diagram for the instrumental setup. The system consists of a nano-reactor system, a SEC system and a CE electrophoretic system with gel filling. A microtee (Upchurch Scientific, Oak Harbor, WA) was used to connect the nano-reactor system, SEC system and the pumping system which consisted of a syringe pump (Kloehn, Las Vegas, NV) and a µLC-500 pump (Isco, Lincoln, NE). A two-position valve (Rheodyne, Rohnert Part, CA) was used to selectively connect one of the pumps to the nano-reactor or the SEC column. A microcross from Upchurch was used to connect the SEC system, gel capillary electrophoretic system and the syringe pump. The syringe pump was equipped with a 25 µl syringe with a resolution of 0.52 nl per step. An 8-position multiposition valve (Valco International, Houston, TX) was used in conjunction with the syringe pump to allow the selection of different solutions to pump through the reactor capillary for cleaning or to the microcross for CE separation.

Nano-reactor system. The nano-reactor was constructed with layers of brass sheet (7.5 × 2.5 × 0.025 cm^3) (Small Parts Inc., Milami Lakes, FL) and a Kapton insulated flexible heater (7.5 × 2.5 cm^2, 2 W/cm^2, resistively heated) (Omega Engineering, Stamford, CT) as shown in Figure 2. A thermal epoxy (Delta Bond 155, Wakefield Engineering, Wakefield, MA) was used to bond the brass sheet and the flexible heater together. This nano-reactor has a very small thermal mass since the total thickness excluding the capillary reactor is ~300 µm so that it allows the temperature to change rapidly. The length (7.5 cm) of the heater allowed the use of eight (9-mm spacing is the standard 8 × 12-tray format) or 16 capillaries (4.5 mm spacing for the 384-tray format) for simultaneous reactions. A 10-cm long, 360 µm o.d. and 75 µm i.d. capillary was placed in between two brass sheets. A silicone heat sink compound (Radio Shack) was applied onto the capillary surface and in

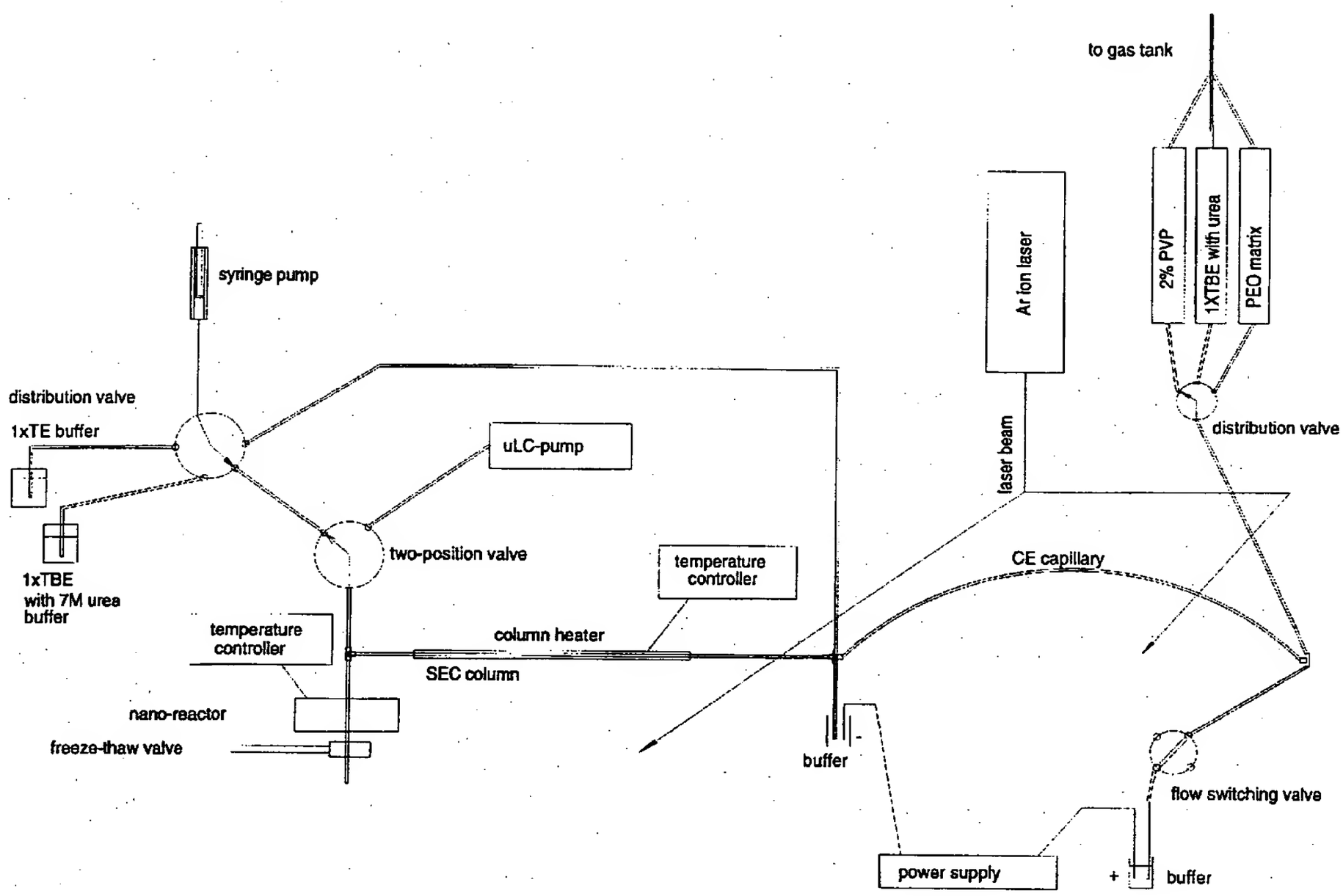

Figure 1. Schematic diagram of the experimental setup. Samples and reagents are introduced from the lower left and transported from left to right for reaction and then separation.

between the brass sheets to ensure proper heat transfer. For 75 μm i.d. capillary, the 23 mm reaction length corresponds to ~110 nl maximum reaction volume. One could simply change to different inner diameters to accommodate different reaction volumes. A 0.08 cm diameter bare K type thermocouple (Omega Engineering), was used to monitor the temperature of the nano-reactor. This thermocouple was inserted into a 250 μm i.d., 360 μm o.d. capillary in which water was filled and both ends of the capillary were sealed. The thermocouple was positioned ~0.5 cm from the reaction capillary. A PID temperature controller (CN77300; Omega) was used to set the temperature profile for cycle sequencing. A computer was used to communicate with the temperature controller to effect the temperature change and duration. In this way ±0.5°C accuracy can be obtained and the heating rate was ~3°C/s. A room-temperature nitrogen gas jet which was controlled by a solenoid valve (Valcor Scientific, Springfield, NJ) was directed to the heater to quickly lower the temperature during the transition from denature to annealing conditions. When ~5 p.s.i. gas pressure was used, ~3°C/s cooling rate can be obtained. A faster cooling rate can be obtained by using higher gas flow rate but slight overshoot may occur.

Fluid manipulation. For capillary cleaning, 250 μl of 1× TE buffer was first pumped through the reaction capillary by the syringe pump. Then 200 nl of 50 mM Tris, 2.5 mM HCl and 2 mM $MgCl_2$ solution was aspirated into the capillary reactor followed by aspiration of 50 nl of cycle-sequencing reaction mixture at 21 nl/s (Fig. 3, left). The Tris solution was used as a buffer zone to isolate the reaction solution from the TE separation buffer since EDTA will interfere with the cycle-sequencing reaction. To add the sample, 500 nl template was placed in a microcentrifuge tube. The tip of the reaction capillary was placed into the bottom of the tube. The syringe pump first aspirated 100 nl of the template solution (Fig. 3, middle) and then dispensed 100 nl (Fig. 3, middle). This mixing procedure (last 2 steps) was repeated 12 times to allow complete mixing of the reaction mixture and the template solution. The bulk of the pre-mix reagents was thus maintained inside the capillary throughout this procedure. After the mixing, the reaction solution was moved up to the nano-reactor by aspirating an additional 180 nl of solution while the capillary tip was still positioned in the template tube.

A freeze/thaw valve, which was described previously (17), was positioned at the entrance end of the reaction capillary to

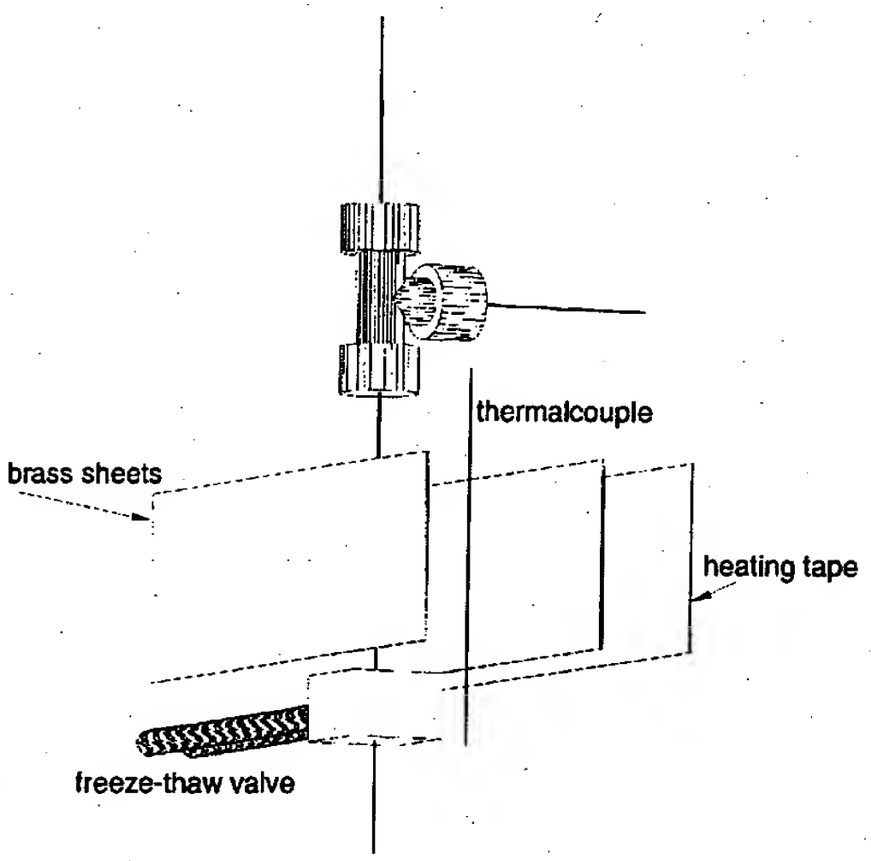

Figure 2. Schematic diagram of the nano-reactor for cycle-sequencing reaction. The capillary runs from bottom to top through the freeze–thaw valve and the microtee.

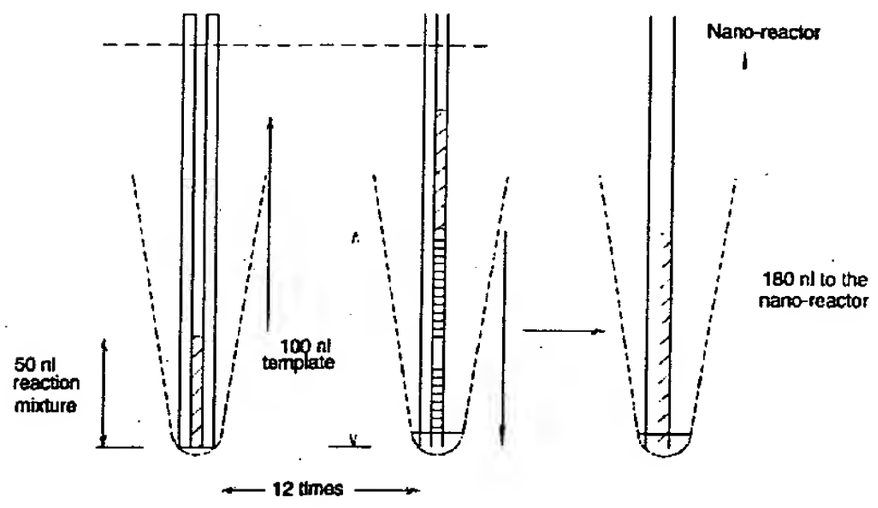

Figure 3. Schematic diagram of the solution mixing procedure. Left: 50 nl of reagent mixture was introduced. Middle: the pump was cycled 12 times to aspirate and then redispense 100 nl of template solution. Right: after such mixing, 180 nl total volume was taken up for reaction.

close it during the cycle-sequencing reaction or SEC separation. During the reaction, TE buffer was flowed through the SEC system to condition the column and to pressurize (>60 p.s.i.) the nano-reactor to eliminate bubble formation.

SEC chromatographic system. The procedure for packing the SEC column with size-exclusion media (Sephadex G-25, Sigma) was described previously (15). The following modification was used to accommodate the requirement for small sample volume. After the particles were fully swollen (hydrated) in 1× TE buffer off-line, a portion of the slurry was transferred by a syringe into a 1/4″ o.d. 3/16″ i.d. PTFE tubing (Cole-Parmer, Vernon Hills, IL). One end of the tubing was connected to a nitrogen gas tank while the other was connected to a 250 μm i.d., 360 μm o.d. capillary. The end of the capillary was connected to an in-line mini microfilter with 2 μm frit (Upchurch). Pressure (60 p.s.i.) was applied to force the Sephadex particles into the capillary column for ~2 h. The column was then inserted into a column heater (CH-1530, Systec, New Brighton, MN). The entrance end of the column was connected to the microtee directly without using any in-line filter. A 30 μm i.d., 360 μm o.d. capillary with 38 cm length was used to connect the SEC column to the injection microcross. The SEC column was heated at 50°C by the column heater during the separation. An ultra-plus μ-LC pump was used to provide 1 μl/min flow rate for SEC separation.

Gel capillary electrophoresis with automatic gel filling system. A 75 μm i.d., 360 μm o.d. capillary with 50 cm effective length was used for gel separation. The injection end of the capillary was connected to the microcross and the other end of the capillary was connected to a microtee, which was connected to the gel filling system. Three reservoirs contained 1× TBE buffer with urea, 2% PVP coating solution and PEO sieving matrix, respectively. A nitrogen gas tank was used to pressurize the reservoirs. A distribution valve (Upchurch) was used to select the solution to be filled into the CE column. A 200 μm i.d. 360 μm o.d. capillary was used to connect a 180° flow switching valve (Upchurch) to the CE column. This switching valve was closed while the capillary was filling. Pressure (60 p.s.i.) was used to push the buffer (2 min), PVP solution (5 min) and PEO matrix (30 min) into the CE column. After completion of gel matrix filling, the 180° flow switching valve was opened. The TBE buffer was used to push the remaining gel matrix out of the connection tubing and formed a connection between the buffer reservoir and the CE capillary. During the filling cycle, both syringe pump and μ-LC pump were turned on to flush the microcross to ensure no gel matrix remained in the cross.

Detection system. A laser induced-fluorescence detection system was used for both SEC and capillary gel electrophoresis separations. A 15-mW Ar-ion laser (Uniphase, San Jose, CA) was used for the excitation on both separation systems by splitting the laser beam 50/50. Both systems used an uncoated plano-convex lens with 12-mm focal length to focus the laser beams on the capillaries. For SEC separation detection, a 10× microscope objective (Edmund Scientific, Barrington, NJ) was used to collect the fluorescence into a photomultiplier tube (PMT) with the use of a 560 nm long-pass filter to cut off the laser scattering. For capillary gel electrophoresis, another 10× microscope objective was used. A 514 nm notch-plus filter (Kaiser Optical System, Ann Arbor, MI) was used to block the laser scattering. Then a 80/20 beam splitter was used to split the fluorescence into two PMTs for simultaneous monitoring. One PMT (80% splitting ratio) had an additional RG630 long-pass filter. This arrangement allowed base-calling for DNA sequencing (27). All PMTs (R928, Hamamatsu Corp., Bridgewater, NJ) were terminated with 10-kΩ resistors before connecting to a 24-bit A/D interface (PC4350, National Instruments, Austin, TX).

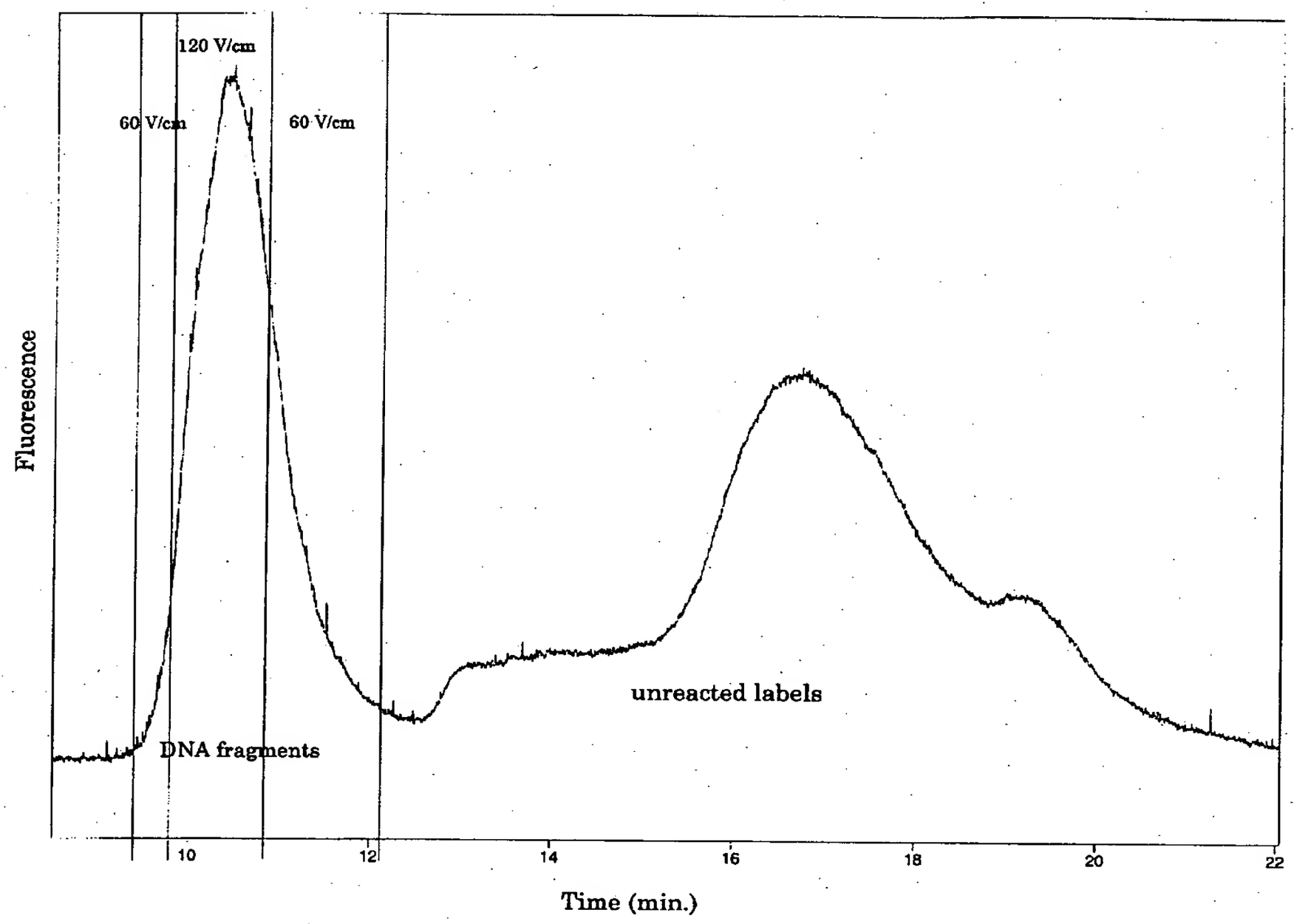

Figure 4. SEC separation of the cycle-sequencing reaction product from the nano-reactor at 50°C with 1 µl/min flow rate. The cycle-sequencing reaction was done in 50 nl reagent volume with 35 cycles. The injection conditions for CE separation are also depicted.

Computer control. A Pentium II 266 MHz computer (Dell, Austin, TX) was used for data acquisition. The temperature controller, cooling valve for the nano-reactor, freeze/thaw valve, syringe pump, µ-LC pump and gel filling system were all controlled by the computer. LabView (version 5.0, National Instruments) was the programming platform for the control software. In-house software was used to call bases by determining the peak intensity ratio of each base from both PMT signals (27).

Operation protocols

After the reaction capillary was cleaned, the reagent mixture was aspirated into the capillary followed by mixing with the template solution with the procedure described previously. The freeze/thaw valve was closed followed by switching the two-position valve (Rheodyne, Rohnert Park, CA) so that 1× TE effluent could flow through the SEC column for conditioning during the cycle-sequencing reaction. Before the reaction was completed, TBE buffer, PVP coating solution and PEO gel were filled into the electrophoretic capillary in preparation for the CE separation. After the cycle-sequencing reaction, the nano-reactor was heated to 95°C for 2 min for denaturing the DNA products. Then the two-position valve was switched back to connect the syringe pump to the nano-reactor. The freeze/thaw valve was opened and allowed the aspiration of an additional 800 nl 1× TE solution to move the reaction products over the microtee. The freeze/thaw valve was closed and the two-position valve was switched back to connect the µ-LC pump to the SEC column to push the reaction products into the SEC column for purification. Fluorescence from the SEC column was monitored. A positive high-voltage was applied at 60 V/cm field strength when the DNA signal appeared at the first detector (~10 s delay time due to the 38 cm long connection capillary). Thirty seconds later, the field strength was increased to 120 V/cm for 60 s followed by a decrease in the field strength to 60 V/cm for the remaining injection period as indicated in Figure 4. The total injection time was ~3 min. Stacking occurs during the entire period. After injection, the two-position valve was switched to disconnect the µ-LC pump and the SEC column to stop the flow of the effluent into the microcross. Otherwise, the dye-labeled terminators will continuously pump through the microcross and subsequently become injected into the CE column. The syringe pump then delivered 1× TBE with 7 M urea buffer to the microcross at a

flow rate of 1.3 µl/min for the CE separation. The field strength for the CE separation was set at 120 V/cm.

RESULTS AND DISCUSSION

One potential problem for cycle sequencing in small inner-diameter capillaries is the large surface-to-volume ratio in which DNA polymerase activity can be inhibited. By adding BSA into the reaction solution, no loss in activity of polymerase was found in as small as 50 µm i.d. capillary. We found that a final concentration of 1× BSA is optimal for these reactions.

By aspirating a dye solution into the capillary with a syringe pump for a fixed time period, one can visually determine how accurately the syringe pump could function. It was found that one can easily aspirate 25 nl of the solution into the 75 µm i.d. capillary with ~10% error. Although only a 10-cm length of capillary was used for the nano-reactor, there was a time delay on the fluid movement due to friction. Therefore, between each syringe pump action, a 6-s waiting period was added to ensure that the fluid completed the motion.

In order to evaluate the mixing procedure, 50 nl (corresponding to 1 cm in length) of concentrated Rhodamine 6G solution was aspirated into the capillary. After mixing with water following the above mixing protocol, the dye solution spread out to ~2 cm. Although this mixing procedure may not provide uniform distribution of the reagent, primer and template across the entire reaction zone, the cycle-sequencing reaction is relatively robust and tolerates such a variation. For further testing, a series of 10, 12, 14 and 16 mixing cycles were used to determine the cycle-sequencing efficiency by observing the fluorescence signal after the SEC separation. It was found that 12 mixing cycles provided the largest DNA signal under these reaction conditions. The initial template concentration also played a role on the reaction efficiency. A range of concentrations from 0.2 to 0.025 µg/µl was used to determine the cycle-sequencing reaction efficiency. It is found that 0.05 µg/µl template concentration provides the highest efficiency.

Figure 4 shows the SEC separation of the cycle-sequencing reaction products by AmpliTaq FS reaction mixture with M13mp18 DNA template. The DNA fragments were completely separated from the dye-labeled terminators. During the cycle-sequencing reaction, the SEC column was flushed with buffer. Because it took ~3 h to finish the total reaction process, a total volume of 180 µl of TE buffer was used (1 µl per min) to clean and condition the column. No cross-contamination between runs was observed in a series of 15 reactions. This SEC column was used for >3 months (60 runs) and maintained similar separation efficiency throughout.

Both ThermoSequenase and AmpliTaq FS have been used successfully for cycle sequencing. Similar reaction efficiency was obtained compared with the use of a commercial hot-air thermal cycler (Idaho Technology, Idaho Falls, ID). The mixing procedure and the reaction efficiency are highly reproducible. The same reaction capillary can be used over a half-year period (80 reactions). Occasionally, the reaction efficiency was reduced after several weeks of operation. One can then simply aspirate 800 nl 1 M NaOH solution into the capillary reactor and then flush with 500 µl of 1× TE buffer to restore the reaction efficiency. No permanent degradation on reaction efficiency has been observed. This is important to automation and long-term operation. By simply flushing the nano-reactor with 250 µl of TE buffer, which corresponded to ~550× column volume, no cross-contamination has been seen for consecutive cycle-sequencing reactions.

Figure 5 shows the electropherogram of the cycle-sequencing products from M13mp18 DNA template after nano-reaction followed by SEC purification with one-wavelength excitation and dual-wavelength detection. Here, 50 nl reaction mix was aspirated initially for cycle-sequencing reaction. No dye-labeled ddNTPs interference can be observed. The success rate is 100% over 15 consecutive runs. Good signal-to-noise and separation resolution were obtained from the electropherogram with a DNA read length of >450 bp using an in-house two-wavelength intensity ratio scheme (27). Future implementation of multi-wavelength base calling will significantly extend the read length. Miniaturization of the injection region will also provide better signal-to-noise and resolution for base calling. It is interesting to see that no denaturing is necessary during the sample injection period. It is possible that when the reaction plug moved over the microtee into the SEC column, sufficient dilution of the reaction plug prevented the DNA from renaturing.

As little as 25 nl reaction mix can be used in this system with slightly compromised read length due to a lower signal-to-noise ratio. The mixing procedure was altered slightly to accommodate the smaller uptake volume. Only 60 nl aspirate–dispense mixing cycle was used. The electropherogram still allowed base calling up to 380 bp.

To reduce the cost of the primers rather than the sequencing reagents, instead of putting the primer into the ready reaction mixture, one can pre-mix the template with the reaction solution. In this case, 0.4 µg of M13mp18 template was added to the ready reaction mix. The primer stock solution was 0.6 µM. The primer was then mixed with the reaction solution later inside the nano-reactor (Fig. 3, middle). Such a protocol will be desirable whenever the primer is the expensive component. In this way, one can simply use a different primer for multiple sequencing to implement, for example, the primer-walking scheme (28). We found no apparent difference in the reaction efficiency and the sequencing separation up to 450 bp in this 'primer-mixing' procedure. However, the signal is reduced significantly (3×) afterwards and no compression peak can be observed.

The use of 500 nl template volume is convenient for standard microvials. If a smaller sample tube such as those used in the 384-tray format is used to hold the reagent solution and a 150 µm o.d. capillary is used as the nano-reactor, only ~150–200 nl solution volume is necessary for the operation in Figure 3. In the reaction solution, ~60% vol (30 out of 50 nl) is the expensive ready reaction pre-mix solution containing dye-labeled terminators and enzyme. In the standard reaction protocol, 4 µl of pre-mix solution is used in a total of 10 µl reaction volume. Over 130-fold reduction in reagent used is thus achieved. Even compared to the state-of-the-art 1 µl reaction volume protocol (11–13) over 13-fold reduction is obtained.

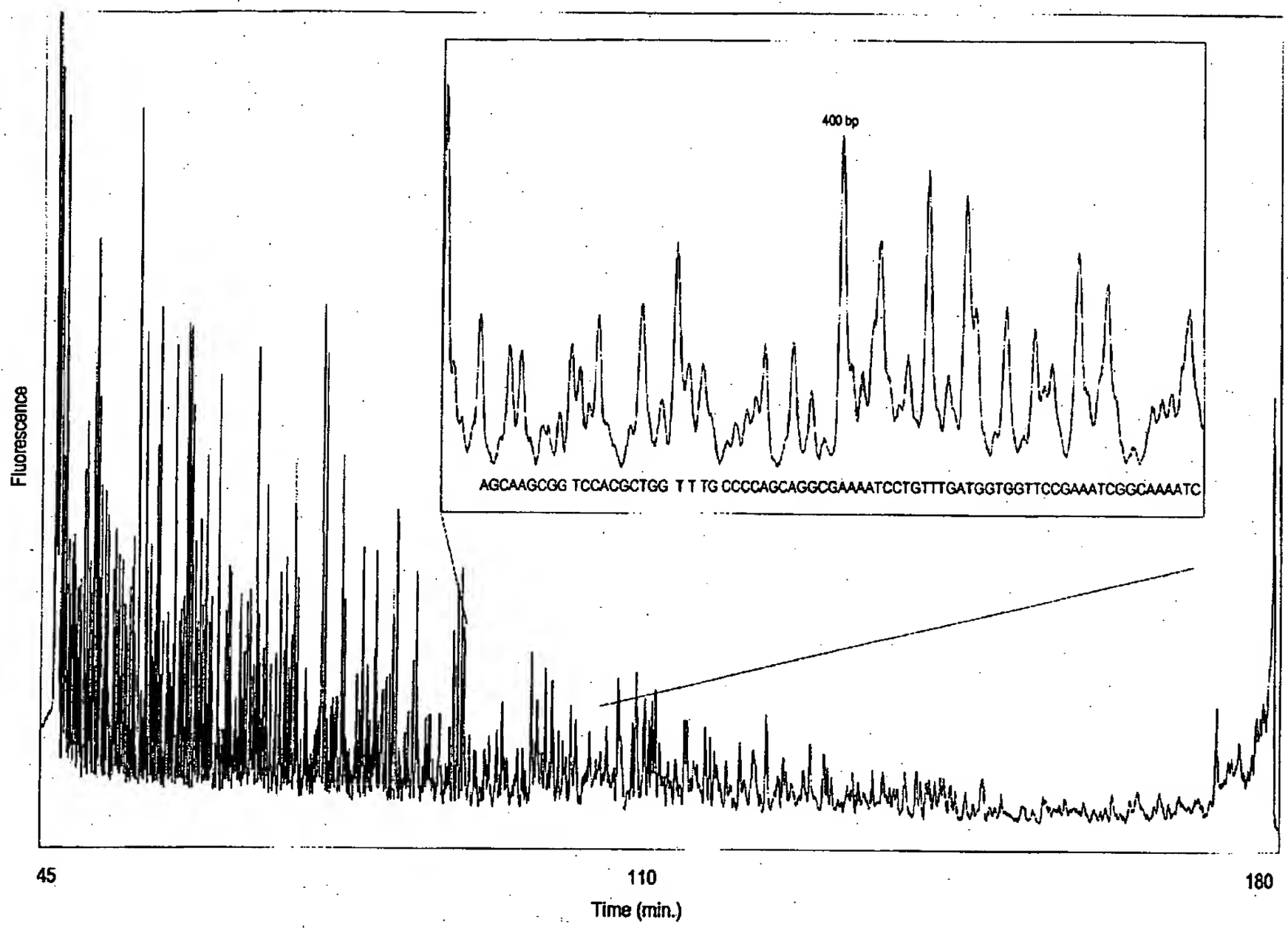

Figure 5. Electropherogram of DNA fragments after cycle-sequencing reaction for M13mp18 ssDNA template amplified by AmpliTaq polymerase inside the nano-reactor with purification by SEC followed by on-line injection into the CE column. Only one wavelength channel was shown for clarity. A field strength of 120 V/cm with 50 cm effective length was used for CE separation. The injection scheme is shown in Figure 4.

CONCLUSIONS

A simple but reliable nano-reactor for cycle sequencing has been demonstrated. The coupling of on-line purification and CE separation provides a substantial real saving in reagent cost. In addition, the system (except for the preparation of stock solutions) is completely automated with computer control. This system is ready to be expanded to the capillary array operation. The substitution of two 10″ × 1″ flexible heaters, which are available commercially, will allow cycle-sequencing reactions in a 96-capillary array. A single larger size syringe (50 µl) in the syringe pump will allow dispensing or aspirating all 96 capillary reactors simultaneously through a manifold (17–19). The same µ-LC pump can provide a high enough flow rate for 96 SEC separations.

The turnaround time for this integrated system was ~6 h including 3 h for cycle-sequencing reaction, 10 min for the SEC separation and 3 h for CE separation. If an additional syringe pump is employed to provide the flow during CE separation, the next round of cycle-sequencing reaction can be performed while the first round of separation is taking place. This will cut the turnaround time by half. The cycle-sequencing time can in principle be reduced to 30 min (16). Here we are limited by the heating rate and the cooling rate of our heater. The separation speed can also be increased to allow sequencing in 30 min (29). By changing the reaction conditions, one can sequence from a single bacterial colony (19) to eliminate the time needed for reincubation. This will be a major advance towards high-speed high-throughput DNA sequencing. Finally, this setup will also allow the performance of PCR reaction followed by CE analysis, even starting directly from biological samples such as blood (18).

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